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USE OF MONOCLONAL ANTIBODIES TO ENUMERATE SPIROCHETES AND IDENTIFY TREPONEMA DENTICOLA IN DENTAL PLAQUE OF CHILDREN, ADOLESCENTS AND YOUNG ADULTS

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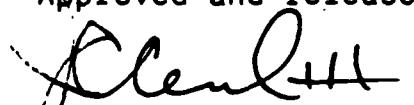

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Use of monoclonal antibodies to enumerate spirochetes and identify *Treponema denticola* in dental plaque of children, adolescents and young adults

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Identification of spirochetes in dental plaque is difficult. Not all spirochetes can be cultured and microscopic analysis based on darkfield or phase optics cannot determine the genus and species of individual bacterial cells. The purpose of this study was to use monoclonal antibodies in an immunoenzyme technique to stain spirochetes in dental plaque. Separate mAb were used to estimate total spirochetes and relative numbers of 2 distinct types of *Treponema denticola*. Plaque samples were collected from 40 subjects grouped by age. Results showed that older subjects are more likely to have spirochetes, to have more spirochetes and to have more diverse populations or spirochetes than younger subjects. Our studies suggest that *T. denticola* may be the first treponeme to colonize the primary dentition, that *T. denticola* appears to comprise a major proportion of all spirochetes at all ages and that two distinct serotypes of *T. denticola* are found to coexist in plaque from most children.

Key words: monoclonal antibody; spirochete; *Treponema denticola*; plaque

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Spirochetes can be found in dental plaque associated with apparently healthy periodontal structures of both children (1) and adults (15). However, spirochetes are found most often and in greatest numbers in association with gingival inflammation and periodontal disease in both children (1, 10, 17) and adults (6, 7, 11). These associations are based on observations derived from experiments using culture techniques (7) or microscopic analyses using darkfield (1, 2, 7, 10, 17) or phase optics (9). *In vitro* isolation and culture permit identification of genus and species but are restricted in application to those spirochetes that are isolated by chance and that will replicate in the laboratory. Culture methods underestimate the variety of spirochetes in plaque because many spirochetes observed microscopically cannot be grown *in vitro* (7, 16). Microscopic approaches permit the rapid identification of morphologic types and relative numbers of spirochetes in plaque but cannot be used to identify either genera or species (10).

Simonson et al. recently produced monoclonal antibodies (mAb) against *Treponema denticola* (14) and showed that the amount of *T. denticola* in plaque increased directly in proportion to the severity of periodontal disease (12). This work suggested that mAb could also be used to detect and identify specific treponemes in dental plaque using conventional immunostaining methods. The purpose of this investigation was to use mAb to estimate relative numbers of spirochetes present in dental plaque and to estimate the relative numbers of discrete types of *T. denticola* within this population.

Material and methods

Subjects

Forty subjects (2–25 years, 24 male, 16 female) were included in the study. All subjects had 4 first molars (primary or permanent) and 4 maxillary and mandibular incisors (primary or permanent). No subject had periodontal disease or any systemic disease and no subject had received any systemic drug therapy, including antibiotics, within 3 months. No subject acknowledged a history of any spirochetal disease. Subjects had not received a dental prophylaxis within 6 months.

Subjects were divided into 4 groups of 10 each according to age: Group A, ages 2–4 (deciduous dentition); Group B, ages 5–11 (mixed dentition); Group C, ages 12–15 (permanent dentition, around puberty); Group D, ages 16–25 (young adults). Subjects were grouped without regard to gender since gender has not been associated with distribution of oral spirochetes in young subjects (10, 17).

Dental plaque

Plaque was collected from gingival sulci at mesiobuccal line angles of all first

Table 1. Expected reactivity of monoclonal antibodies

mAb	Source (Reference)	Reactivity
C2–1	S. Lukehart, Seattle, WA (8)	*Borrelia recurrentis* *Leptospira interrogans* *T. denticola* *T. hyodysenteriae* *T. pallidum* subsp. *pallidum* *T. pallidum* subsp. *pertenue* *T. phagedenis* (Reiter) *T. vincentii*
TD-II	L. Simonson, Great Lakes, IL (14)	*T. denticola* ATCC 33521
TD-III[1]	L. Simonsen	*T. denticola* ATCC 33520 *T. denticola* ATCC 35404
H9–1	S. Lukehart (5, 8)	*T. pallidum* subsp. *pallidum* *T. pallidum* subsp. *pertenue*
Bg	L. Simonson (13)	*Bacteroides gingivalis*

[1]Unpublished observations

molars, from the facial surfaces of maxillary right central incisors, and from facial surfaces of mandibular right central incisors. The curette blade was cleaned with a new alcohol swab between each site collection. Thus, although plaque collection was standardized, in order to obtain enough plaque from young children with healthy gingiva, the 6 samples for each patient were pooled into 0.5 ml phosphate-buffered saline (PBS), pH 7.0. In order to maintain a uniform technique, all 6 sites were pooled for all subjects.

Each plaque sample was gently dispersed with a Pasteur pipette and 50 μl of plaque suspension was placed on each of approximately 10 glass slides per subject. Slides were air-dried and stored at 0 C.

Bacteria

Cultivable treponemes, including *T. denticola* (ATCC 33520, 33521, 35404, 35405), *Treponema pectinovorum* (ATCC 33763), *Treponema phagedenis* biotype Reiter (Dr. J. N. Miller, UCLA), *Treponema refringens* (Dr. R. George, Centers for Disease Control, Atlanta, GA), *Treponema scoliodontium* (J. N. Miller), *Treponema socranskii* (ATCC 35536), and *Treponema vincentii* (J. N. Miller), were grown in spirolate broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with 10% sterile, heat-inactivated (56°C, 30 min) normal rabbit serum (GIBCO Laboratories, Grand Island, NY) at 34°C. *Bacteroides gingivalis* (ATCC 33277) was grown in Wilkin's-Chalgren broth (Oxoid, Basingstoke, Hampshire, UK) supplemented with 5 μg/ml hemin and 0.3 μg/ml menadione. *Treponema pallidum* subsp. *pallidum* (Nichols strain) were grown in rabbits, extracted in PBS and stored at −70 C. Bacteria were washed 3 times and resuspended to their original volumes in PBS supplemented with 0.02 M magnesium chloride (13) and 10 μl of each suspension dried onto glass slides. Slides were stored at 0°C.

Table 2. Observed specificities of monoclonal antibodies

	C2–1	TD-II	TD-III	H9–1	Bg
B. gingivalis	−	−	−	−	+
T. denticola ATCC 33520	+	−	+	−	−
T. denticola ATCC 33521	+	+	−	−	−
T. denticola ATCC 35404	+	−	+	−	−
T. denticola ATCC 35405	+	−	+[a]	−	−
T. pallidum	+	−	−	+	−
T. pectinovorum	+	+[a]	−	−	−
T. phagedenis	+	−	−	−	−
T. refringens	+	−	−	−	−
T. scoliodontium	+	−	−	−	−
T. socranskii	+	−	−	−	−
T. vincentii	+	−	−	−	−

[a]These cross-reactions were not observed in whole-cell ELISA (Simonson, unpublished observations).

Monoclonal antibodies

Sources and expected reactivities of each mAb used in this investigation are listed in Table 1. The specificities of each mAb have been described previously (5, 8, 13, 14). Monoclonal antibody TDII, IAA11 (serovar "B") will be referred to hereafter as TD-II; TDIII, 1IIBB2 (serovar C) as TD-III; and BgII,VF9/2d as Bg.

Immunochemistry

Dried plaque and control bacteria on slides were fixed in 100% acetone at 4 C for 10 min, air-dried, and hydrated in PBS just before staining. Reagents were applied in 50-μl volumes at room temperature in a humidor and slides were rinsed and immersed in PBS for 5 min between each step except as noted. Normal rabbit serum blocking solution (10% NRS) (Zymed Laboratories, catalog #95–6561) was applied for 10 min, drained and, without washing, mAb diluted 1:2 in blocking solution was added for 60 min. Plaque sample slides for each subject were processed with mAb C2–1, H9–1, and Bg. Plaque samples reacting with C2–1 were processed with TD-II and TD-III in subsequent experiments. Prediluted biotinylated-rabbit anti-mouse antibodies (polyspecific for IgA, IgG, and IgM, H and L chains) (Zymed Laboratories, catalog #61–6440) were applied for 10 min. Strepavidin-alkaline phosphatase conjugate (1:100 dilution in PBS) (Zymed Laboratories, catalog # 43–4322) was added for 10 min and slides were washed as before. Substrate (p-nitrophenyl phosphate) and chromogen (fast blue) solution (Zymed Laboratories, catalog #00–2204) was added for 15 min. Slides were washed in distilled water, blotted and coverslips applied with a glycerine-based mounting solution.

Slides were viewed at × 1000 magnification. All blue-stained spirochetes in 5 non-overlapping high-power fields were counted and an average was obtained for each mAb and each subject. A mean relative number, ±one standard deviation, of spirochetes per high-powered field stained by each mAb in each group was computed.

Controls

One plaque slide per subject per mAb analysis was treated with PBS instead

Table 3. Mean relative number of bacteria per viewing field stained with mAb

Group (Age)	mAb			
	C2-1	TD-II	TD-III	Bg
A (2–4)	1.20±2.41[a]	0.70±0.98	1.52±2.11	0.00±0.00
B (5–11)	28.74±38.71	4.36±5.77	8.35±11.44	0.36±0.76
C (12–15)	9.66±7.69	1.72±1.45	6.00±6.93	0.04±0.13
D (16–25)	10.48±9.47	2.14±1.51	4.56±3.39	0.08±0.25

[a]Mean (±standard deviation) relative number of bacteria per viewing field stained with mAb.

of mAb as a negative (reagent) control. H9–1 was used as an additional negative (treponeme mAb) control since this mAb reacts only with a determinant only present on *T. pallidum* subspecies.

T. phagedenis biotype Reiter and *T. denticola* ATCC 33521 were used as positive (bacteria) controls for monoclonals C2–1 and TD-II, respectively. The same 2 treponemes were used as negative controls when PBS was used instead of mAb on control bacteria slides.

B. gingivalis ATCC 33277 and *T. denticola* ATCC 33520 served as positive controls for monoclonals Bg and TD-III, respectively.

Statistical analysis

The Newman-Keuls multiple comparison technique for one-way analysis of variance was used to compare differences between means among the 4 age group for each mAb. Significance was set at the 0.05 level. Pearson's product-moment coefficient of correlation was used to determine relationships between relative numbers and prevalence of bacteria and age. Significance was determined with *t*-tests with alpha ≤ 0.05.

Results

Specificity of monoclonal antibodies

Each mAb was tested against each control bacteria (Table 2). C2–1 reacted, as expected, with every treponeme tested, including *T. scoliodontium* and *T. socranskii*, but not with *B. gingivalis*. Td-II reacted with *T. denticola* ATCC 33521, and, unexpectedly, with *T. pectinovorum*. Td-III bound *T. denticola* ATCC 33520 and *T. denticola* ATCC 35404 as predicted, but also attached to *T. denticola* ATCC 35405. The specificities of Td-II and Td-III are non-overlapping. H9 1 did not react with any oral treponeme tested.

Enumeration of spirochetes in plaque

Negative control slides were uniformly negative, demonstrating that rabbit antibodies did not crossreact with human antibodies in plaque and that neither mAb H9–1, biotin nor strepavidin was bound nonspecifically by plaque components. Negative controls also indicated that plaque spirochetes did not express endogenous alkaline phosphatase activity under these conditions.

Spirochetes were detected with C2–1 in each age group (Table 3). Group B (ages 5–11 years) had the highest mean relative number of spirochetes, 28.74± 38.71 and Group A (2–4 years) the lowest, 1.20±2.41. There was a difference ($P<0.05$) between means of Group B and Group A but no other differences between means were significant. Relative numbers of spirochetes detected with C2–1 showed a relationship with age ($r=0.50$, $P=0.01$; Table 5). The percentages of subjects with spirochetes per group were 50%, 70%, 70% and 90% for Groups A, B, C and D, respectively (Table 4). The prevalence of spirochetes was correlated with age ($r=0.96$, $P=0.05$). Relative numbers of spirochetes identified with C2–1 for each subject are shown in Fig. 1A. A marked increase in relative spirochete counts can be seen at approximately 9–14 years. Three 9-year-old subjects (100, 83 and 34.8 spirochetes/field, respectively) and one 10-year-old (60/field) had more spirochetes than could be plotted on the scale in Fig. 1A.

Identification of *T. denticola*

Plaque samples stained with TD-II demonstrated the presence of treponemes sharing antigens with certain strains of *T. denticola* and with *T. pectinovorum* (Table 3). The mean relative numbers of treponemes detected with TD-II ranged from 0.70±0.98 (Group A) to 4.36±5.77 (Group B). There was no significant difference between means among age groups and no relationship between relative numbers detected with TD-II and age (Table 5). *T. denticola* serovar "B" prevalences ranged from 50% in Group A to 90% in Group D (Table 4) and showed a relationship with age ($r=0.96$, $P=0.05$; Table 5). Fig. 1B shows the relative number of treponemes detected with TD-II in relation to age. Most subjects had treponemes reactive with TD-II in dental plaque. The 3 subjects with highest counts also had high C2–1 counts.

Mean relative counts of treponemes stained with TD-III ranged from 1.52±2.11 to 8.35±11.44 (Groups A and B, respectively) (Table 3). Again, no significant differences between means were noted among age groups and there was no relationship between relative numbers and age (Table 5). Percentages of subjects in groups positive with TD-III ranged from 40% to 90% (Table 4) and appeared to correlate with increasing age ($r=0.97$, $P=0.05$; Table 5). However, Fig. 1C shows the relative number of treponemes detected with TD-III in relation to age. There appeared to be increased numbers of subjects with TD-III (+) trepc :mes around 9–13 years due to high numbers from 5 subjects, 2 of which also had high C2–1 counts; otherwise, numbers

Table 4. Percentage of subjects per group reactive with mAb

Group	C2-1	TD-II	TD-III	Bg
A	50%	50%	40%	0%
B	70%	70%	60%	30%
C	70%	70%	60%	10%
D	90%	90%	90%	10%

Table 5. Statistical analysis of the relationship between the presence of mAb-labelled treponemes and age of subjects

Correlation	r^a	p^b
C2-1 count & age[c]	0.50	0.01
TD-II count & age	0.08	NS
TD-III count & age	0.10	NS
Prevalence of stained bacteria:		
T. denticola[d] & age	−0.40	NS
C2-1+& age	0.96	0.05
TD-II+& age	0.96	0.05
TD-III+& age	0.97	0.05
Bg+& age	0.06	NS

[a]Pearson's product-moment correlation coefficient; [b]*t*-test; [c]Excluding 4 scores of Group B that were greater than mean±3 SD; [d]Sum of subjects with bacteria stained with TD-II and/or TD-III/total number of subjects.

of TD-III (+) spirochetes appeared to be constant from young children through young adults. Thus, it is unlikely that there is a significant correlation between TD-III and age.

T. denticola (TD-II and/or TD-III) was found in plaque of every child with spirochetes (C2–1+) and, for 4/5 children with spirochetes in Group A, *T. denticola* appeared to be the major, if not sole component of the spirochetes population defined by C2–1. After approximately 4–5 years, *T. denticola* represented approximately 40–80% of plaque spirochetes.

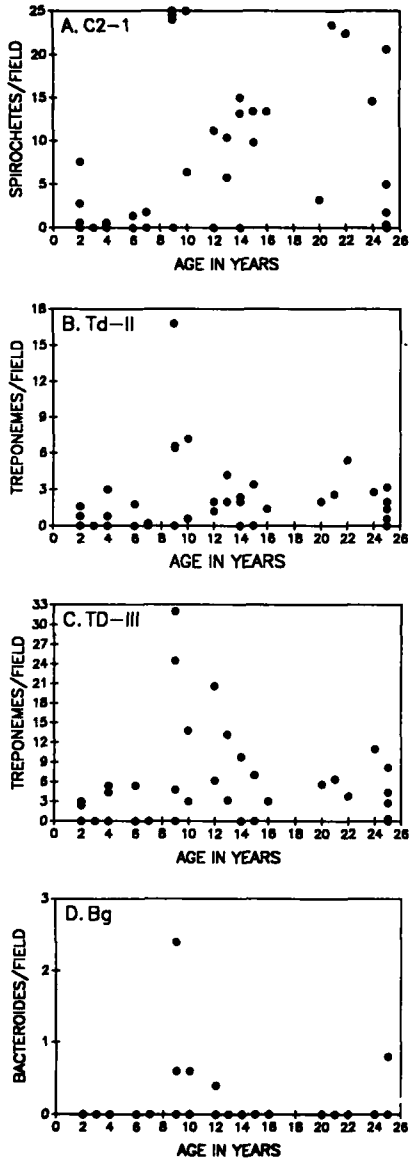

Fig. 1. Relative number of bacteria[a] detected with mAb.

[a]Scatter plot of each subject for each mAb; A = C2–1, which detects a determinant common to all spirochetes; B = TD-II, which detects *T. denticola* serovar "B"; C = TD-III, which detects *T. denticola* serovar C; and D = Bg, which detects *B. gingivalis*. Three 9-year-old and 1 10-year-old subject had off-scale counts of 100, 83, 34.8 and 60, respectively, with C2–1 and are shown at the top in Fig. 1A using contrived values of 24, 24.5 and 25. Note that scale on that *y* axis changes in each figure.

The mean relative numbers of bacteria reacting with Bg ranged from 0.00 ± 0.00 (Group A) to 0.36 ± 0.75 (Group B) (Table 3). There were no significant differences between mean values among age groups and no correlation was found between relative numbers and age (Table 5). The prevalences for Groups A, B, C and D, respectively, were 0%, 30%, 10% and 10% (Table 4) and showed no significant relationship with age (Table 5). Fig. 1D shows that most subjects did not appear to have reactive *B. gingivalis* in dental plaque. However, 3/4 subjects who had very high numbers of spirochetes (C2–1+) and TD-II (+) treponemes also had high counts with Bg.

Discussion

This study employed monoclonal antibodies in a sensitive immunocytochemical technique to stain spirochetes within plaque samples and to identify specific treponemes within each sample. The method permits staining of single bacterial cells and, with the appropriate mAb, identification of genus, species and serotype.

The use of mAb C2–1, previously reported to react with an antigen common to *Borrelia, Leptospira* and *Treponema* (8), permitted the detection of spirochetes in 70% of subjects in this study, including half of the youngest group, ages 2–4 years. Since all subjects in this study had clinically healthy gingiva, these observations support the conclusion that spirochetes are part of the normal oral flora (10, 15), even in young children.

The use of mAb C2–1 in this study also supports conclusions derived from other studies (2, 7, 9) that older children tend to have more oral spirochetes than younger children. The frequency of C2–1+ samples in our studies was equivalent or slightly higher than the prevalence reported in other studies for similarly grouped children (2, 7, 9) and these differences were greatest in younger subjects. For example, Mackler & Crawford (9) reported approximately 26% and Loesche (7) reported 40% of children ages 3–5 had spirochetes in plaque, compared with 50% observed in this study. When subjects aged 5 through 11 were assessed with C2–1, 70% demonstrated spirochetes, while prior studies with similarly grouped ages reported 66% (2) and 50% (7). Another study reported much higher percentages than the present investigation, finding spirochetes in practically all Tanzanian children aged 6–12 (10), perhaps reflecting a geographic or cultural difference in spirochete prevalence. 70% of subjects aged 12–15 demonstrated the presence of spirochetes with C2–1 but in a study by Ashley et al. (1), 88.7% of subjects 14–15 years of age were found to have spirochetes. If 14- and 15-year-old subjects were isolated from the present study, a similar percentage would be appararent. As expected, the group of 16- to 25-year-old subjects demonstrated the highest prevalence of spirochetes (90%). Finally, it should be stressed that although this assay can detect as few as one bacterial cell on a slide, sampling error and random distribution of cells within samples prevent efforts to quantify in an absolute sense.

This is the first study to use monoclonal antibodies to determine the presence of a defined genus and species of treponeme in dental plaque of children. Half (5/10) of 2- to 4-year-old subjects had treponemes detectable with TD-II, and 4 of these also had treponemes detectable with TD-III. As these 2 mAb have non-overlapping specificities, it is evident that these young children had 1 or 2 serotypes of *T. denticola* in their dental plaque. However, there were so few spirochetes in plaque from these young children that it is only safe to say that *T. denticola* is probably the first treponeme to colonize dental plaque and that it is the predominant treponeme present in dental plaque of young children.

The proportion of children with spirochetes in plaque increased with age but, after about 5 years of age, 20–46% of the total spirochete population (detected by C2–1) did not react with *T. denticola* antibodies. Furthermore, whereas total relative numbers of spirochetes (detected with C2–1) increased in a significant relationship with age, populations of *T. denticola* did not. Therefore, these data suggest that a more diverse population of spirochetes emerges in the environment of healthy periodontal tissues as children age.

The prevalence and relative numbers of *B. gingivalis* were very low among the 4 age groups in this study and had no apparent relationship with age. Although *B. gingivalis* is known to be uncommon in the oral flora of children (3, 4, 17, 18), the present study provided evidence of *B. gingivalis* in dental plaque as early as 9 years of age in some subjects and in the presence of clinically healthy gingiva. These findings emphasize the utility of mAb for detecting very low numbers of bacteria in random plaque samples of children.

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18. SUPPLEMENTARY NOTES

Oral Microbiol Immunol 1991: 6: 97-101

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Monoclonal Antibody; Spirochetes; Treponema denticola; Plaque

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Identification of spirochetes in dental plaque is difficult. Not all spirochetes can be cultured and microscopic analysis based on darkfield or phase optics cannot determine the genus and species of individual bacterial cells. The purpose of this study was to use monoclonal antibodies in an immunoenzyme technique to stain spirochetes in dental plaque. Separate mAb were used to estimate total spirochetes and relative numbers of 2 distinct types of Treponema denticola. Plaque samples were collected

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from 40 subjects grouped by age. Results showed that older subjects are more likely to have spirochetes, to have more spirochetes and to have more diverse populations or spirochetes than younger subjects. Our studies suggest that T. denticola may be the first treponeme to colonize the primary dentition, that T. denticola appears to comprise a major proportion of all spirochetes at all ages and that two distinct serotypes of T. denticola are found to coexist in plaque from most children.